Article 34

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New Claims

1. A method for detection of pathogenic enterobacteria in a sample comprising PCR amplification of DNA isolated from said sample using a set of oligonucleotide primer pairs allowing differentiation of at least two groups of pathogenic E.coli strains by amplification of a virulence factor/toxin gene characteristic for the respective group of the pathogenic E. coli strains.

2. The method according to claim 1 wherein the set of oligonucleotide primer pairs comprises two or more primer pairs selected from

- a primer pair that hybridises to a gene encoding heat labile toxin, or heat stabile toxin for amplification of a DNA sequence characteristic for enterotoxigenic E. coli;

- a primer pair that hybridises to a gene encoding heat stabile toxin for amplification of a DNA sequence characteristic for enteroaggregative E. coli;

- a primer pair that hybridises to the pCVD432 plasmid for amplification of a DNA sequence characteristic for enteroaggregative E.coli;

- a primer pair that hybridises to the inv-plasmid for amplification a DNA sequence contained in enteroinvasive E.coli;

- a primer pair that hybridises to the EAF plasmid, or the eae gene for amplification of a DNA sequence characteristic for enteropathogenic E.coli;

- a primer pair that hybridises to the genes encoding shiga-like toxin sltI or sltII for amplification of a DNA sequence characteristic for enterohemorrhagic E.coli.

3. The method according to claim 2 wherein

the primer pair that hybridises to the gene encoding heat labile toxin characteristic for enterotoxigenic E. coli is

LT-1: 5' GCG TTA CTA TCC TCT CTA TGT G 3' and

LT-2: 5' AGT TTT CCA TAC TGA TTG CCG C 3' ;

the primer pair that hybridises to the gene encoding heat stabile toxin characteristic for enterotoxigenic E. coli is

ST-1: 5′ TCC CTC AGG ATG CTA AAC CAG 3′ and

ST-2a: 5′ TCG ATT TAT TCA ACA AAG CAA C 3′ ;

the primer pair that hybridises for the gene encoding heat stabile toxin characteristic for enteroaggregative E. coli is

EASTI-1: 5′ AAC TGC TGG CTA TGT GGC TGG 3′ and

EASTI-2: 5′ TGC TGA CCT GCC TCT TCC ATG 3′ ;

the primer pair which hybridises to the pCVD432 plasmid is

EA-1: 5′ CTG GCG AAA GAC TGT ATC ATT G 3′ and

EA-2: 5′ TAA TGT ATA GAA ATC CGC TGT T 3′ ;

the primer pair which hybridises to the inv-plasmid is

EI-1: 5′ TTT CTG GAT GGT ATG GTG AGG 3′ and

EI-2: 5′ CTT GAA CAT AAG GAA ATA AAC 3′ ;

the primer pair which hybridises to the EAF plasmid is

EP-1: 5′ CAG GGT AAA AGA AAG ATG ATA AG 3′ and

EP-2: 5′ AAT ATG GGG ACC ATG TAT TAT C 3′ ;

the primer pair which hybridises to the eae gene is

EPeh-1: 5′ CCC GGA CCC GGC ACA AGC ATA AG 3′ and

EPeh-2: 5′ AGT CTC GCC AGT ATT CGC CAC C 3′ ;

the primer pair which hybridises to the gene encoding shiga-like toxin SltI is

SltI-1: 5′ ATG AAA AAA ACA TTA TTA ATA GC 3′ and
SltI-2: 5′ TCA CYG AGC TAT TCT GAG TCA AGC 3′;

the primer pair which hybridises to the gene encoding shiga-like toxin SltII is

SltII-1: 5′ ATG AAG AAG ATR WTT RTD GCR GYT TTA TTY G 3′ and
SltII-2: 5′ TCA GTC ATW ATT AAA CTK CAC YTS RGC AAA KCC 3′

wherein W is A/T, R is A/G, D is A/G/T, Y is C/T and K is G/T.

4. The method according to claims 1 to 3 wherein a polymerase having additional 5′-3′ exonuclease activity is used for the amplification of DNA, and an oligonucleotide probe labelled at the most 5′ base with a fluorescent dye and at the most 3′ base with a fluorescent quencher dye which hybridises within the target DNA is included in the amplification process; said labelled oligonucleotide probe being susceptible to 5′-3′ exonuclease degradation by said polymerase to produce fragments that can be detected by fluorogenic detection methods.

5. The method according to claim 4 wherein the labelled oligonucleotide probe is specific for the respective virulence factor/toxin gene to be detected.

6. The method according to claim 5 wherein

the labelled oligonucleotide probe is specific for the detection of heat labile toxin characteristic for enterotoxigenic E. coli;

the labelled oligonucleotide probe is specific for the detection of heat stabile toxin characteristic for enterotoxigenic E. coli;

the labelled oligonucleotide probe is specific for the detection of heat stabile toxin characteristic for enteroaggregative E. coli;

the labelled oligonucleotide probe is specific for the detection of pCVD432 plasmid;

the labelled oligonucleotide probe is specific for the detection of the inv-plasmid;

the labelled oligonucleotide probe is specific for the detection of the EAF-plasmid;

the labelled oligonucleotide probe is specific for the detection of the eae gene;

the labelled oligonucleotide probe is specific for the detection of shiga-like toxin SltI gene;

the labelled oligonucleotide probe is specific for the detection of shiga-like toxin SltII gene.

7. The method according to claim 6 wherein

the labelled oligonucleotide probe for the detection of heat labile toxin characteristic for enterotoxigenic E. coli is

5′ AGC TCC CCA GTC TAT TAC AGA ACT ATG 3′ ;

the labelled oligonucleotide probe for the detection of heat stabile toxin characteristic for enterotoxigenic E. coli is

5′ ACA TAC GTT ACA GAC ATA ATC AGA ATC AG 3′;

the labelled oligonucleotide probe for the detection of heat stabile toxin characteristic for enteroaggregative E. coli is

5′ ATG AAG GGG CGA AGT TCT GGC TCA ATG TGC 3′;

the labelled oligonucleotide probe for the detection of pCVD432 plasmid is

5′ CTC TTT TAA CTT ATG ATA TGT AAT GTC TGG 3′;

the labelled oligonucleotide probe for the detection of the inv-plasmid is

5′ CAA AAA CAG AAG AAC CTA TGT CTA CCT 3′

the labelled oligonucleotide probe for the detection of the EAF-plasmid is

5′ CTT GGA GTG ATC GAA CGG GAT CCA AAT 3′;

the labelled oligonucleotide probe for the detection of the eae gene is

5′ TAA ACG GGT ATT ATC AAC AGA AAA ATC C 3′;

the labelled oligonucleotide probe for the detection of shiga-like toxin SltI gene is

5′ TCG CTG AAT CCC CCT CCA TTA TGA CAG GCA 3′;

the labelled oligonucleotide probe for the detection of shiga-like toxin SltII gene is

5′ CAG GTA CTG GAT TTG ATT GTG ACA GTC ATT 3′.

8. The method according to claims 4 to 7 wherein the fluoroscent reporter dye is 6-carboxy-fluoroscein, tetrachloro-6-carboxy-fluoroscein, or hexachloro-6-carboxy-fluoroscein, and the fluorescent

quencher dye is 6-carboxytetramethyl-rhodamine.

9. The method according to claims 1 to 8 wherein the amplification process comprises 35 PCR cycles at a $MgCl_2$ concentration of 5.2 mmol, an annealing temperature of 55 °C and an extension temperature of 65 °C.

10. A set of primer pairs useful for PCR amplification of DNA of pathogenic enterobacteria allowing differentiation of at least two different groups of pathogenic E. coli strains by amplification of a virulence factor/toxin gene characteristic for the respective group of the pathogenic E.coli strains.

11. The set of primer pairs according to claim 10 comprising two or more primer pairs selected from

a primer pair that hybridises to a gene encoding heat labile toxin, or heat stabile toxin of enterotoxigenic E. coli;

a primer pair that hybridises to a gene encoding heat stabile toxin of enteroaggregative E. coli;

a primer pair that hybridises to the pCVD432 plasmid of enteroaggregative E. coli;

a primer pair that hybridises to the inv-plasmid of enteroinvasive E. coli;

a primer pair that hybridises to the EAF plasmid, or the eae gene of enteropathogenic E. coli;

a primer pair that hybridises to the gene encoding shiga-like toxin sltI or sltII of enterohemorrhagic E. coli.

12. The set of primer pairs according to claim 11 wherein

the primer pair which hybridises to the gene encoding heat labile toxin of enterotoxigenic E. coli is

LT-1: 5′ GCG TTA CTA TCC TCT CTA TGT G 3 and
LT-2: 5′ AGT TTT CCA TAC TGA TTG CCG C 3′ ;

the primer pair which hybridises to the gene encoding heat stabile toxin of enterotoxigenic E. coli is

ST-1: 5′ TCC CTC AGG ATG CTA AAC CAG 3′ and
ST-2a: 5′ TCG ATT TAT TCA ACA AAG CAA C 3′ ;

the primer pair which hybridises to the gene encoding heat stabile toxin of enteroaggregative E. coli is

EASTI-1: 5′ AAC TGC TGG GTA TGT GGC TGG 3′ and

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EASTI-2: 5′ TGC TGA CCT GCC TCT TCC ATG 3′ ;

the primer pair which hybridises to the pCVD432 plasmid is

EA-1: 5′ CTG GCG AAA GAC TGT ATC ATT G 3′ and
EA-2: 5′ TAA TGT ATA GAA ATC CGC TGT T 3′ ;

the primer pair which hybridises to the inv-plasmid is

EI-1: 5′ TTT CTG GAT GGT ATG GTG AGG 3′ and
EI-2: 5′ CTT GAA CAT AAG GAA ATA AAC 3′ ;

the primer pair which hybridises to the EAF plasmid is

EP-1: 5′ CAG GGT AAA AGA AAG ATG ATA AG 3′ and
EP-2: 5′ AAT ATG GGG ACC ATG TAT TAT C 3′ ;

the primer pair which hybridises to the eae gene is

EPeh-1: 5′ CCC GGA CCC GGC ACA AGC ATA AG 3′ and
EPeh-2: 5′ AGT CTC GCC AGT ATT CGC CAC C 3′ ;

the primer pair which hybridises to the shiga-like toxin sltI gene is

SltI-1: 5′ ATG AAA AAA ACA TTA TTA ATA GC 3′ and
SltI-2: 5′ TCA CYG AGC TAT TCT GAG TCA AGC 3′ ;

the primer pair which hybridises to the shiga-like toxin sltII is

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SltII-1: 5' ATG AAG AAG ATR WTT RTD GCR GYT TTA TTY G 3'

and

SltII-2: 5' TCA GTC ATW ATT AAA CTK CAC YTS RGC AAA KCC 3'

wherein W is A/T, R is A/G, D is A/G/T, Y is C/T and K is G/T.

13. A set of labelled oligonucleotide probes useful for detection of pathogenic enterobacteria by TaqMan™-PCR being specific for virulence factor/toxin genes of pathogenic E. coli strains.

14. The set of probes according to claim 13 comprising

a labelled oligonucleotide probe specific for the detection of heat labile toxin characteristic for enterotoxigenic E. coli;

a labelled oligonucleotide probe specific for the detection of heat stabile toxin characteristic for enterotoxigenic E. coli;

a labelled oligonucleotide probe specific for the detection of heat stabile toxin characteristic for enteroaggregative E. coli;

a labelled oligonucleotide probe specific for the detection of pCVD432 plasmid;

a labelled oligonucleotide probe specific for the detection of the inv-plasmid;

a labelled oligonucleotide probe specific for the detection of the EAF-plasmid;

a labelled oligonucleotide probe specific for the detection of the eae gene;

a labelled oligonucleotide probe specific for the detection of shiga-like toxin SltI gene;

a labelled oligonucleotide probe specific for the detection of shiga-like toxin SltII gene.

15. The set of probes according to claim 14 wherein

the labelled oligonucleotide probe for the detection of heat labile toxin characteristic for enterotoxigenic E. coli is

5′ AGC TCC CCA GTC TAT TAC AGA ACT ATG 3′;

the labelled oligonucleotide probe for the detection of heat stabile toxin characteristic for enterotoxigenic E. coli is

5′ ACA TAC GTT ACA GAC ATA ATC AGA ATC AG 3′;

the labelled oligonucleotide probe for the detection of heat stabile toxin characteristic for enteroaggregative E. coli is

5′ ATG AAG GGG CGA AGT TCT GGC TCA ATG TGC 3′;

the labelled oligonucleotide probe for the detection of pCVD432 plasmid is

5′ CTC TTT TAA CTT ATG ATA TGT AAT GTC TGG 3′;

the labelled oligonucleotide probe for the detection of the inv-plasmid is

5′ CAA AAA CAG AAG AAC CTA TGT CTA CCT 3′

the labelled oligonucleotide probe for the detection of the EAF-plasmid is

5′ CTT GGA GTG ATC GAA CGG GAT CCA AAT 3′;

the labelled oligonucleotide probe for the detection of the eae gene is

5′ TAA ACG GGT ATT ATC AAC AGA AAA ATC C 3′;

the labelled oligonucleotide probe for the detection of shiga-like toxin SltI gene is

5′ TCG CTG AAT CCC CCT CCA TTA TGA CAG GCA 3′;—

the labelled oligonucleotide probe for the detection of shiga-like toxin SltII gene is

5' CAG GTA CTG GAT TTG ATT GTG ACA GTC ATT 3'.

16. A kit useful for diagnosing an enterobacteria infection in samples derived from a living animal body, including a human, by TaqMan™-PCR method comprising a set of primer pairs according to claims 10 to 12 and a set of oligonucleotide probes according to claims 13 to 15.

17. Use of the method according to claims 1 to 9 for diagnosing an enterobacteria infection in a sample derived from a living animal body, including a human, or for the detection of an enterobacteria contamination of consumables, such as meat, milk and vegetables.